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Mito-nuclear discordance in the phenotypically variable Andean hummingbirds *Coeligena bonapartei* and *Coeligena helianthea* (Trochilidae)

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The interplay among evolutionary mechanisms like gene flow and selection may result in discordant signals between mitochondrial DNA, nuclear markers and phenotypes. The Andean hummingbirds *Coeligena bonapartei* and *Coeligena helianthea* showed differentiation in the gene *ND2* which is discordant with plumage coloration but consistent with geography. We analysed complete mitochondrial genomes of individuals from *Coeligena bonapartei bonapartei*, *Coeligena bonapartei consita*, *Coeligena helianthea helianthea*, and *Coeligena helianthea tamai* to inform their evolutionary history. We found genetic structure despite low genetic differentiation among these populations. Phylogenetic and network analyses based on mitogenomes showed a northern vs. southern differentiation pattern which is discordant with the relationships based on nuclear markers and the coloration phenotypes (serving as a basis for taxonomy). Mitogenomes of the two nominate subspecies are indistinguishable, suggesting incomplete lineage sorting or introgression, while those of *C. b. consita* and *C. h. tamai* are more similar to each other than they are to their respective nominate subspecies. Our results indicate that various evolutionary mechanisms drove the divergence in phenotypes, and nuclear and mitochondrial genomes of *Coeligena* hummingbirds, playing out over a complex biogeographic scenario likely involving periods of isolation and secondary contact. We outline hypotheses to be tested with future analyses of genome-wide variation.

ADDITIONAL KEYWORDS: Andes – introgression – mitogenomes – recent divergence – relict lineages.

INTRODUCTION

Mitochondrial genes and genomes (mtDNA) are expected to reflect the evolutionary history of lineages, particularly when divergence is mainly driven by genetic drift (commonly in geographic isolation) (Moore, 1995; Ballard & Whitlock, 2004). Because the effective population size of the mitochondrial genome (n) is lower than that of the nuclear genomes ($2n$), the former evolves faster than the latter when drift is the leading mechanism of evolution (Avise *et al.*, 1987; Ballard & Whitlock, 2004). In such cases the phylogenies of mitochondrial markers are more or equally informative than those inferred from nuclear markers, and they agree with phenotypic differentiation which is commonly used to distinguish species and populations. However, in some cases the phylogenies of mitogenomes disagree with those of the nuclear genome (Mito-nuclear discordance) and with variation in phenotypes, suggesting that various evolutionary

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mechanisms may have acted on the differentiation and divergence of the lineages involved (Toews & Brelsford, 2012). For example, divergent selection acting on nuclear markers or purifying selection acting on mitochondrial markers may result in phylogenies where nuclear regions show more differentiation than those generated using mitogenomes. Also, gene flow between divergent lineages may facilitate the introgression and replacement of the mitogenome via selection, and lead to different lineages with the same mitogenome despite nuclear and phenotypic markers showing clear differentiation (Irwin *et al.*, 2009; Rheindt *et al.*, 2011; Toews & Brelsford, 2012). Therefore, concordance or discordance among the phylogenies inferred from mitogenomes, nuclear markers and phenotypes are informative about the evolutionary history of lineages.

In birds, phenotypic characters such as those derived from morphology, plumage and songs are commonly used to compare populations, to study speciation and to inform taxonomy (Edwards *et al.*, 2005; Remsen, 2005). Morphological measurements may provide evidence of barriers to gene flow (Cadena *et al.*, 2018), and visual and acoustic signals are key phenotypes for species delimitation because they are involved in species recognition and reproductive isolation (Roulin, 2004; Price, 2008; Uy *et al.*, 2009). Studies on Neotropical birds often show concordant differentiation between phenotypic characters and mitochondrial markers among lineages (e.g. Lovette *et al.*, 2010; Sedano & Burns, 2010; Gutiérrez-Pinto *et al.*, 2012; Ribas *et al.*, 2012; Valderrama *et al.*, 2014; Winger & Bates, 2015), or cases where mtDNA is highly structured despite little variation in plumage (e.g. D'Horta *et al.*, 2013; Valderrama *et al.*, 2014; Cadena *et al.*, 2019; Chesser *et al.*, 2020; Gutiérrez-Zuluaga *et al.*, 2021). In contrast, cases documenting species with marked phenotypic differences but little mitochondrial differentiation are comparably scarce (Campagna *et al.*, 2012; Naka *et al.*, 2012; Lougheed *et al.*, 2013; Cortes-Rodriguez *et al.*, 2016; Luna *et al.*, 2017).

In hummingbirds (Trochilidae), concordance in variation between mtDNA and plumage coloration among species and populations appears to be the norm [*Adelomyia* (Chaves *et al.*, 2007); *Amazilia* (Jiménez & Ornelas, 2016); Trochilidae (McGuire *et al.*, 2008); *Amazilia* (Ornelas *et al.*, 2014); *Coeligena* (Parra *et al.*, 2009); *Eugenes* and *Lamprolaima* (Zamudio-Beltrán & Hernández-Baños, 2015, 2018)]. Variation in coloration and mtDNA often coincide even when phenotypic variation is subtle among hummingbirds, such as in the colour of the crown, gorget or tail [*Metallura* (Benham & Witt, 2016); *Campylopterus curvipennis* (González *et al.*, 2011); *Antocephala* (Lozano-Jaramillo *et al.*, 2014); *Lampornis* (Ornelas *et al.*, 2016); *Amazilia* (but see Rodríguez-Gómez & Ornelas, 2015); *Oreotrochilus* (Sornoza-Molina *et al.*, 2018)]. There are, to our knowledge, two documented cases of hummingbirds showing low mtDNA differentiation with marked coloration differentiation (i.e. differences in colour in several plumage patches; Parra, 2010; Eliason *et al.*, 2020), both occurring in the high Andes. One case involves two species of *Metallura* metaltails [*Metallura theresiae* and *Metallura eupogon* (García-Moreno *et al.*, 1999; Benham *et al.*, 2015)], and the other, two species of starfrontlets in the genus *Coeligena* (Parra *et al.*, 2009; Palacios *et al.*, 2019) which we focus on in this study.

The golden-bellied starfrontlet *Coeligena bonapartei* (Boissonneau, 1840) and the blue-throated starfrontlet *Coeligena helianthea* (Lesson, 1838) inhabit the northern Andes of Colombia and Venezuela (Fig. 1A). The nominate subspecies are sympatric in the southern part of their ranges in the Cordillera Oriental of Colombia, whereas the subspecies *C. b. consita* and *C. h. tamai* are allopatric to the north in the Serranía de Perijá and Tamá Massif, respectively. These species are strikingly different in structural plumage coloration (Eliason *et al.*, 2020; Sosa *et al.*, 2020): *C. bonapartei* is greenish with fiery golden underparts, whereas *C. helianthea* is blackish with a rose belly and aquamarine rump (Fig. 1). Despite their markedly different phenotypes, *C. bonapartei* and *C. helianthea* show low genetic differentiation in a mitochondrial gene (*ND2*) and in some nuclear markers [the Melanocortin 1 Receptor *MC1R* gene and regions flanking ultra-conserved elements (UCEs); Palacios *et al.*, 2019]. Phylogenetic analyses of the *ND2* mitochondrial gene also suggested that *C. b. consita* and *C. h. tamai* are more closely related to each other than either is to their respective nominate subspecies, indicating that *ND2* variation better reflects geography than phenotype or taxonomy.

In this study, we obtained and studied in detail mitochondrial genomes of individuals from four populations of the morphologically variable *C. bonapartei* and *C. helianthea* to gain insight into the evolutionary history of these lineages by describing patterns of genetic variation within and among lineages, and by comparing phylogenies derived from complete mitogenomes, mitochondrial genes and nuclear markers. We also assessed the possible functional implications of the substitutions among *Coeligena* mitogenomes. Finally, we discussed the implications of our findings in the evolutionary history of these populations and the mechanisms likely acting on their divergence.

MATERIAL AND METHODS

Samples and sequencing

We sampled 46 individuals of *C. bonapartei* and *C. helianthea* (22 of the former and 24 of the latter, Supporting Information, Table S1), representing the

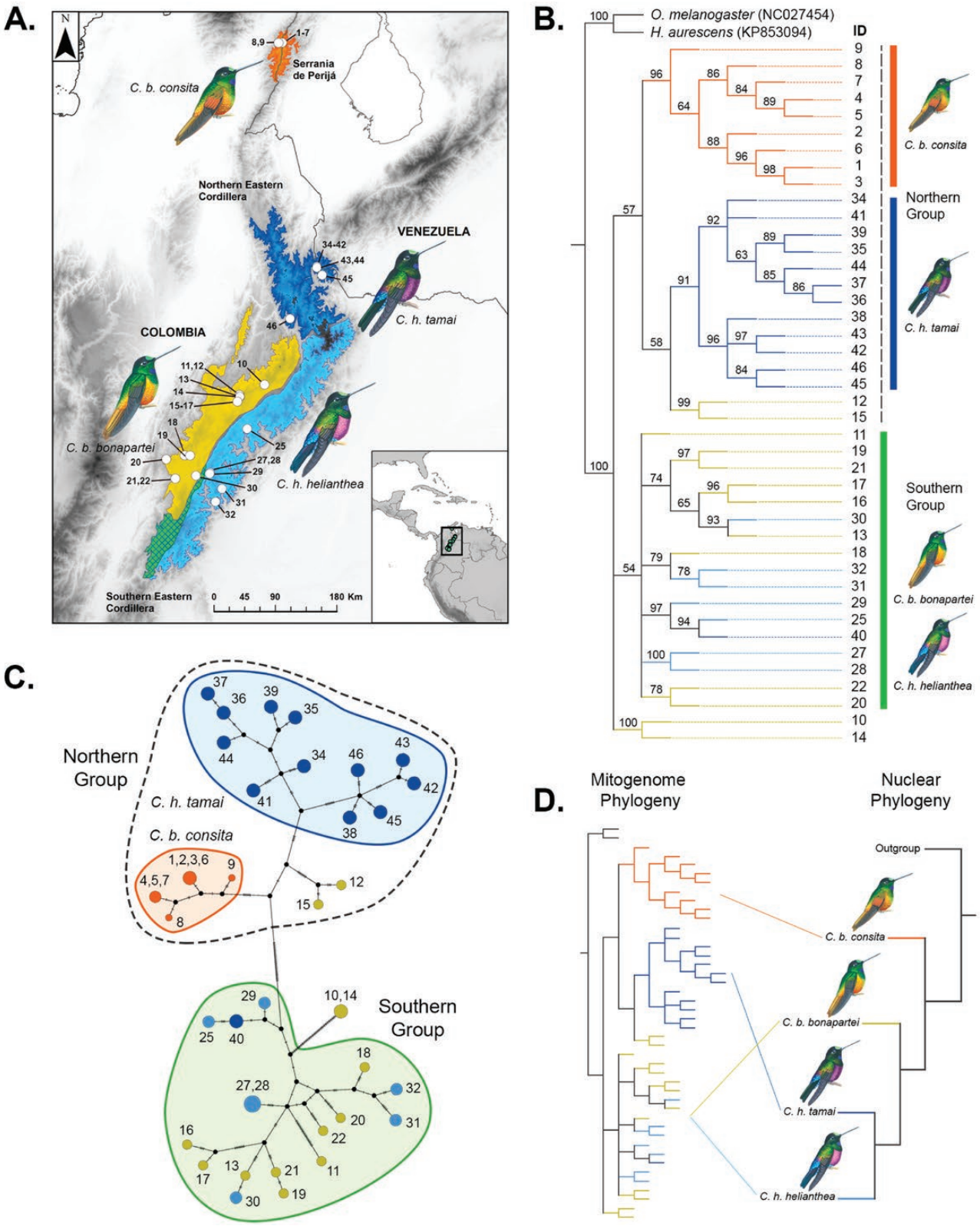

Figure 1. Mitogenome phylogeny and haplotype network of *C. bonapartei* and *C. helianthea* samples support two main (northern and southern) groups, which are discordant with the phylogenetic relationships based on nuclear markers and with the phenotypes (plumage coloration and associated taxonomy). A, sampling and distribution of *C. bonapartei* and *C.*

subspecies *C. b. bonapartei*, *C. b. consita*, *C. h. helianthea* and *C. h. tamai*. Taxon identities were assigned by inspecting specimens in museum collections or by geography. Because previous work indicated that populations from the Mérida Cordillera of Venezuela often referred to as *C. bonapartei* (subspecies *Coeligena bonapartei eos*) are genetically divergent from other populations in the complex (Palacios *et al.*, 2019), we did not consider them in this study. Muscle tissue samples from vouchered specimens were obtained from the collections of the Instituto Alexander von Humboldt (IAvH) and the Museo de Historia Natural de la Universidad de los Andes (ANDES). We evenly sampled subspecies and sex for both *C. bonapartei* and *C. helianthea*.

We extracted total genomic DNA using a standard phenol/chloroform method and Phase-Lock Gel tubes, followed by a standard cleaning protocol employing magnetic beads. We prepared 46 Illumina TruSeq Nano DNA-enriched libraries following the manufacturer's protocol for low-throughput configuration and 550 bp insert size. We quantified the libraries using a Qubit fluorometer. Normalizing, pooling and sequencing were carried out at the Genomics Facility of the Institute of Biotechnology at Cornell University. Sequencing was performed using two lanes of a NexSeq 500 obtaining 150 bp paired-end reads. We checked read quality using Fastqc (Andrews, 2010), and removed adapters using AdapterRemoval (Schubert *et al.*, 2016).

Assembly and annotation of mitochondrial genomes

We used the cleaned reads to assemble mitogenomes using MITObim v.1.9.1 (Hahn *et al.*, 2013) with default parameters. We used two alternative assembling strategies based on different baits: (1) two independent assemblies using the complete mitochondrial genomes of *Oreotrochilus melanogaster* and *Heliodoxa aurescens* (GenBank NC027454 and KP853094, respectively) as baits, and (2) a third assembly using the *ND2* gene sequence as bait for each individual—or a related one—available from a previous work (Palacios *et al.*, 2019). We expected that the mitogenome-bait strategy would allow us to recover more complete mitogenome sequences because during initial iterations reads would map to different sites on the reference mitogenome and this would allow extension from multiple edges. In turn, we expected that the gene-bait strategy would enable us to identify structural changes in genomes because it would allow extension only from the two edges of the gene; however, it would likely be susceptible to recovering incomplete sequences when reads did not overlap.

We compared the results from each strategy to determine the sequence and structure of mitogenomes of *C. bonapartei* and *C. helianthea*. In addition, we mapped the read-pool obtained from the mitogenome-bait strategy against the mitogenome sequence obtained from the gene-bait strategy using the 'map to reference assemble' tool in Geneious 9.1.5 (http://www.geneious.com; Kearse *et al.*, 2012). We used these map-to-reference assemblies to close gaps in sequences, to check the number of repetitions at the end of the control region (see Results), and to verify assigned nucleotides in each sequence at polymorphic sites, therefore producing high quality mitogenomes. We aligned and edited mitochondrial genomes using ClustarO (Sievers *et al.*, 2011) and manually in Geneious, and annotated them using MITOS beta version (http://mitos2.bioinf.uni-leipzig.de/index.py) and Geneious. In addition to the alignment of complete mitogenomes, we produced alignments for each protein-coding gene (PCG), and a concatenated alignment of 13 PCGs (*ND1*, *ND2*, *COX1*, *COX2*, *ATP8*, *ATP6*, *COX3*, *ND3*, *ND4L*, *ND4*, *ND5*, *CYTB* and *ND6*) for Population genetics, Phylogenetics, and Amino Acid Change analyses.

Population Genetics, Phylogenetics, and Amino Acid Change analyses

Using the complete mitogenome alignment, we calculated nucleotide diversity (Pi) for all sequences together, separately for *C. bonapartei*, *C. helianthea*, and for each of the four subspecies (*C. b. bonapartei*, *C. b. consita*, *C. h. helianthea*, *C. h. tamai*). We calculated absolute genetic divergence (Dxy) in DnaSP v.6 (Rozas *et al.*, 2017), and relative genetic divergence (F_{ST}) between species and among subspecies assessing significance with 1000 permutations using the R package Hierfstat (Goudet, 2005; R Core Team, 2017).

We examined phylogenetic relationships among individuals based on each of our alignments using

helianthea lineages, the gridded area in the map is where nominate subspecies are sympatric. B, mitogenome phylogeny, numbers on branches are ML-bootstrap values and branch lengths were set to equal. C, mitogenome haplotype network. Note that the mitogenomes of *C. b. consita* and *C. h. tamai* are differentiated, whereas the mitogenomes of *C. b. bonapartei* and *C. h helianthea* are indistinguishable (southern group, shown in green). D, comparison of discordant mitogenome and nuclear phylogenies. Numbers on the locations in the map, on the tips of the tree, and on the haplotype network correspond to individual IDs shown in the Supporting Information (Table S1). Colours correspond to the assigned subspecies *C. b. consita* (orange), *C. b. bonapartei* (yellow), *C. h. helianthea* (light blue) and *C. h. tamai* (dark blue).

maximum-likelihood analysis and computed majority-rule consensus trees in RAxML v.8.2.12 (Stamatakis, 2014). We used the GTR+GAMMA model and multiparametric bootstrapping stopped by the autoMRE criterion. We used the mitochondrial genomes of *Oreotrochilus melanogaster* and *Heliodoxa aurescens* (GenBank NC027454 and KP853094, respectively) as outgroups. We also built a median-joining haplotype network (Bandelt *et al.*, 1999) in PopArt (Leigh & Bryant, 2015). We compared the phylogenetic relationships of the mitogenome of *C. bonapartei* and *C. helianthea* with those derived from nuclear markers (Palacios *et al.*, 2019).

Finally, we assessed whether there are fixed changes in amino acids in the mitochondrial encoded proteins of the lineages of *C. bonapartei* and *C. helianthea* potentially suggestive of selection acting on their mitogenome. We first calculated the number and type of substitutions in each PCG in DnaSP v.6 (Rozas *et al.*, 2017). Then, for each non-synonymous substitution we examined whether the amino acid variants were from different functional groups.

RESULTS

Sequence and structure of the *C. bonapartei* and *C. helianthea* mitogenomes

We recovered very similar assemblies using the mitogenome-bait and the gene-bait strategies. However, using the mitogenome-bait strategy, we observed insertions in some mitogenomes that we did not recover with the gene-bait strategy, and we found minor differences in the length and sequence of the control region between the strategies. We used the read-pool map-to-reference assemblies to resolve discrepancies between assemblies and to review and manually correct nucleotide assignments at variant sites.

We recovered complete mitogenomes for 42 of the 46 samples (excluding IDs 23, 24, 26 and 33 in Supporting Information, Table S1, which we do not consider further because of low quality), with an average coverage of 127.5 X for all genomes (Max. 1555.7, Min. 11.8, see Supporting Information, Table S1 for details, GenBank accession numbers MT341527 to MT341568). The size of the mitochondrial genome of *C. bonapartei* and *C. helianthea* varied from 16 813 bp to 16 859 bp, mainly due to variation in the length of the control regions due to a repetitive motive ('AAAC'). The 42 sequences were identical across 16 560 bp (98.2%), and showed 248 variant sites (1.5%) with 51 positions showing gaps or being ambiguous (0.3%). The mean pairwise identity was 99.7%, and the total GC content was 44.8%. On average, the mitogenomes of *Coeligena* were 86.0% (14 555 bp) identical to those of *O. melanogaster* and 85.6% (14 450 bp) identical to those of *H. aurescens*. The beginning of the control region (~350 bp) was the most difficult part to align between *Coeligena* and the outgroups. The mitogenome structure of *Coeligena* species followed the typical pattern observed in other birds, including hummingbirds, with two rRNAs, 13 PCGs, 22 tRNAs and the control region (Fig. 2).

Genetic divergence and clustering patterns among lineages of *C. bonapartei* and *C. helianthea*

Across the complete mitogenome alignment including all individuals of *C. bonapartei* and *C. helianthea*, we found only 248 variable sites (250 mutations as two sites have three alleles, 1.5% of the genome). Of these variable sites, 89 were singletons and 159 were parsimony-informative sites. Nucleotide diversity was low in the complete alignment (Pi = 0.00247, SD = 0.00013). The least diverse lineage was *C. b. consita* (Pi = 0.00019, only nine polymorphic sites, Table 1), followed by *C. h. helianthea* (Pi = 0.00084, 40 polymorphic sites), *C. h. tamai* (Pi = 0.00124, 98 polymorphic sites) and *C. b. bonapartei* (Pi = 0.00254, 156 polymorphic sites). When we compared groups based on species assignment, i.e., *C. bonapartei* vs. *C. helianthea*, we found low relative genetic differentiation (F_{ST} = 0.076, P = 0.016). However, F_{ST} values were greater when considering the four lineages separately (Table 1). Lineages assigned to the same species showed higher F_{ST} values than lineages assigned to different species (e.g. *C. b. consita* vs. *C. b. bonapartei* F_{ST} = 0.385, P-value < 0.001; *C. h. helianthea* vs. *C. h. tamai* F_{ST} = 0.518, P < 0.001; *C. b. bonapartei* vs. *C. h. helianthea* F_{ST} = 0.083, P = 0.1). All comparisons indicated low absolute genetic divergence (Dxy), supporting overall low differentiation in the mitogenomes of these lineages (Table 1). However, high values of relative genetic divergence (F_{ST}) between lineages of *C. bonapartei* and *C. helianthea* support genetic structure.

Phylogenetic analyses of the mitogenome alignment clustered all sequences of *Coeligena* hummingbirds in a well-supported clade with respect to the outgroups [maximum-likelihood bootstrap (ML-bs) 100, Fig. 1]. Relationships within *Coeligena* show a polytomy comprising: (1) a clade grouping all sequences of *C. b. consita* (ML-bs 97), (2) a clade grouping all but one of the sequences of *C. h. tamai* (ML-bs 90), and (3) the remaining sequences scattered in smaller clades. Phylogenies built with other alignments (each PCG and concatenated PCGs, Supporting Information, Fig. S1) showed lower resolution (i.e. more polytomies or lower support values). In most phylogenies, *C. b. consita* and *C. h. tamai* were clustered together and

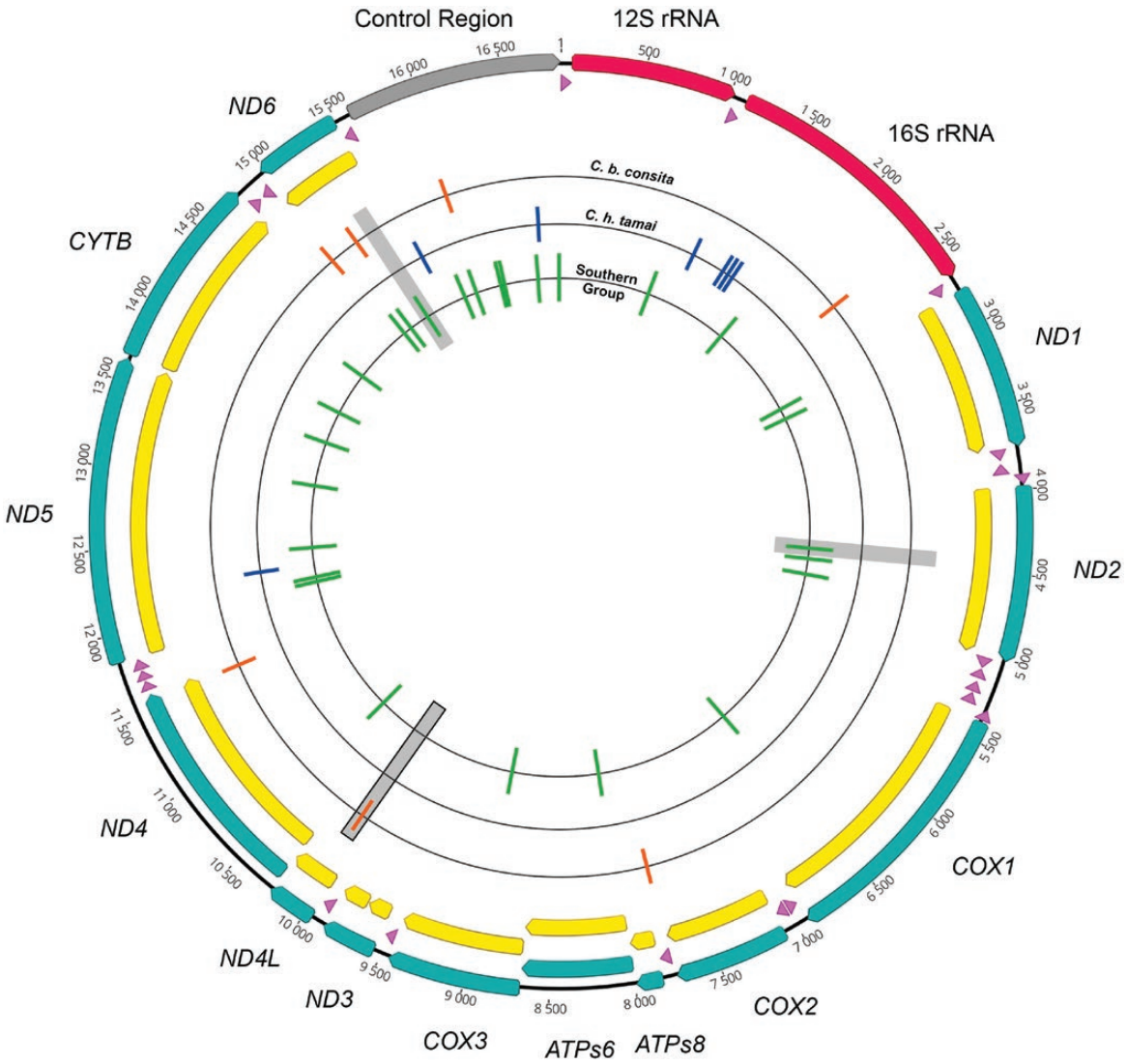

Figure 2. The mitochondrial genome structure of *Coeligena* hummingbirds follows the typical organization of birds: 22 tRNAS (pink), two rRNAs (red), 13 protein-coding genes (PCGs; blue) and the control region (grey). Coding sequences (CDS) are in yellow. Substitutions among the three genetic groups *C. b. consita* (orange), *C. h. tamai* (blue) and the southern group (green) are represented in the inner circles (singletons and intrapopulation variant sites are not shown). Grey boxes indicate the three non-synonymous substitutions found among the genetic groups, the box with black edges indicates the only substitution involving a change between amino acids with different functional features.

not with their nominate subspecies, but the support values for this group were lower than 80% in most phylogenies except for the control-region phylogeny (ML-bs 88).

As with phylogenies, in the median-joining haplotype network all sequences of *C. b. consita* clustered together (Fig. 1); all sequences but one of *C. h. tamai* clustered in another group which was close to, but distinguishable from, two sequences of *C. b. bonapartei* (ID 12 and 15); and the remaining sequences clustered together in a third group (Fig. 1). The network showed that sequences of *C. b. consita* and *C. h. tamai* are more similar to each other than to *C. b. bonapartei* and *C. h. helianthea*. Interestingly, two individuals of *C. b. bonapartei* (ID 10 and 14, same haplotype) were highly divergent from all other individuals. *Coeligena bonapartei consita* was the lineage with the lowest number of haplotypes (four among nine individuals), whereas the number of haplotypes was similar to the number of individuals in the other lineages: 12 haplotypes in *C. b. bonapartei* (13 individuals), six in *C. h. helianthea* (seven individuals) and 13 in *C. h. tamai* (13 individuals).

Based on the findings above, we redefined genetic groups for additional analyses as: (1) a northern

Table 1. Population genetic statistics and measures of genetic divergence. Nucleotide diversity Pi is lower in *C. b. consita* and *C. h. tamai* than in the nominate subspecies. Absolute genetic divergence Dxy is low yet relative divergence F_{ST} is high across comparisons. Genetic groups are derived from the clustering analyses and are marked as 'Gen' in the table

Population genetic statistics

Population	**No. of Seq**	**No. of variants**	**Pi**	**Tajima's D**	**Tajima's D *P*-value**
C. bonapartei	22	171	0.00249	-0.44	0.351
C. helianthea	20	133	0.00218	-0.09	0.481
C. b. consita	9	9	0.00019	-0.05	0.500
C. b. bonapartei	13	156	0.00254	-0.68	0.273
C. h. helianthea	7	40	0.00084	-0.75	0.274
C. h. tamai	13	98	0.00124	-1.54	0.057
C. b. consita Gen	9	9	0.00019	-0.05	0.500
C. h. tamai Gen	12	54	0.00089	-0.76	0.251
Northern group Gen	23	92	0.00120	-0.76	0.242
Southern group Gen	17	100	0.00180	-1.63	0.043

Measures of genetic divergence

Population 1	Population 2	F_{ST}	F_{ST} *P*-value	Dxy
C. bonapartei	*C. helianthea*	0.076	0.0160	0.0026
C. b. consita	*C. b. bonapartei*	0.385	0.0010	0.0032
C. b. consita	*C. h. helianthea*	0.764	0.0010	0.0032
C. b. consita	*C. h. tamai*	0.402	0.0010	0.0017
C. b. bonapartei	*C. h. helianthea*	0.083	0.1069	0.0019
C. b. bonapartei	*C. h. tamai*	0.317	0.0010	0.0034
C. h. helianthea	*C. h. tamai*	0.518	0.0010	0.0033
Northern group Gen	Southern group Gen	0.514	0.0010	0.0035
C. b. consita Gen	*C. h. tamai* Gen	0.502	0.0010	0.0016

group comprising all sequences of *C. b. consita*, all sequences of *C. h. tamai* except ID 40, and two sequences of *C. b. bonapartei* (ID 12 and 15); and (2) a southern group including most sequences of the nominate subspecies *C. b. bonapartei* and *C. h. helianthea* (except the highly divergent ID 10 and 14) and the remaining sequence of *C. h. tamai* (ID 40). We also considered separately the groups of *C. b consita* and *C. h. tamai* (excluding ID 40). There were only 27 substitutions (0.16%) yet high relative genetic divergence (F_{ST} = 0.513, *P*-value < 0.001, Table 1) between the northern and southern genetic groups. Likewise, between *C. b. consita* and *C. h. tamai* there were 14 substitutions (0.083%) and relative genetic divergence was high (F_{ST} = 0.502, *P*-value < 0.001). All other 118 parsimony-informative sites are variable within populations, thus they are not considered sustitutions because they are no fixed among populations.

Phylogenies inferred using nuclear markers (UCEs and SNPs from the whole genome) show that *C. b. consita* was the first branch to diverge in the group, whereas *C. b. bonapartei* is the sister taxon of the *C. helianthea* group, within which *C. h. helianthea* and *C. h. tamai* are reciprocally monophyletic (Fig. 1; Palacios *et al.*, 2019; Palacios, 2020). Because the mitogenomes of *C. b. bonapartei* and *C. h. helianthea* are indistinguishable, and the mitogenomes of *C. b. consita* and *C. h. tamai* cluster together, the phylogenetic relationships derived from nuclear markers are discordant with the phylogenetic relationships inferred using the mitochondrial genomes.

Given a substitution rate of 0.00256 substitutions per site per lineage per million years (s/s/l/Myr) for the complete mitogenome of birds (Eo & DeWoody, 2010), we roughly calculated that the northern and southern mitochondrial groups diverged around 310 000 years ago, and that *C. b. consita* and *C. h. tamai* diverged around 160 000 years ago. Based on 13 PCGs plus the two rRNAs and a substitution rate of 0.00164 s/s/l/Myr (mean rate for Apodiformes; Arcones *et al.*, 2019), estimates of divergence times are similar yet slightly older: 380 000 years ago between the northern and southern mitochondrial groups, and 180 000 years ago between *C. b. consita* and *C. h. tamai*.

Functional amino acid changes

Of the total 248 variant sites, 160 were in PCGs, 88 variant sites were in rRNAs (six sites in 12S rRNA, 20 sites in 16S rRNA), 11 sites in tRNAs, five sites in inter-gene spacers and 46 sites in the control region. Among the 160 variant sites in PCGs, 123 corresponded to synonymous changes and 38 to non-synonymous changes. Most non-synonymous changes were singletons (23 sites) or varied within populations (11 sites). Of the remaining four non-synonymous changes, one was shared between one individual of *C. b. bonapartei* and one individual of *C. h. tamai* (T↔C position 269 in *ND5*). Thus, only three non-synonymous changes corresponded to substitutions between genetic groups. Of them, one change in *ND2* and one in *ND6* were fixed differences between the northern and the southern groups (G↔A position 475 in *ND2*, and G↔A position 112 in *ND6*), but these substitutions do not imply evident functional changes because both amino acids involved (valine and isoleucine) are aliphatic, nonpolar and neutral. Finally, one non-synonymous substitution between *C. b. consita* and all other sequences (A↔G position 145 in *ND4*) implies a functional change in amino acids because whereas *C. b. bonapartei*, *C. h. helianthea* and *C. h. tamai* have the aliphatic, nonpolar alanine, *C. b. consita* has the hydroxyl-containing, polar, threonine. Note that this change is not between the two main mitogenome groups because *C. h. tamai* has the variant of the southern group at this position.

DISCUSSION

We found that the mitochondrial genomes of *C. bonapartei* and *C. helianthea* show low genetic differentiation. Mitogenomes of 42 individuals from four populations of these species were very similar (98.2% identical) and absolute genetic divergence (Dxy) was low. However, relative genetic divergence (F_{ST}) between genetic groups was high, and phylogenetic analyses grouped the mitogenomes of *C. b. consita* and *C. h. tamai* together instead of placing them with their corresponding nominate subspecies. Moreover, mitogenomes of *C. b. bonapartei* and *C. h. helianthea* were indistinguishable from each other. These results agree with the previous patterns based solely on the *ND2* gene (Palacios *et al.*, 2019), and imply there is discordance with the groupings derived from plumage coloration (and associated taxonomy) and with the phylogenetic relationships inferred from nuclear markers (Palacios *et al.*, 2019; Palacios, 2020). The mitogenome variation agrees better with the current distribution of the subspecies, considering that *C. b. consita* and *C. h. tamai* occur to the north (Serranía de Perijá and the north of the Cordillera Oriental, respectively), whereas both nominate subspecies occur to the south along each slope of the cordillera.

Based on the *ND2* gene, the clade formed by *C. bonapartei* and *C. helianthea* diverged from *C. b. eos* around 310 000 years ago, and the northern and southern groups comprising the four lineages diverged around 240 000 years ago (Palacios *et al.*, 2019). The latter estimate is more recent than our calculations of the divergence between the northern and southern groups at *c.* 310 000 (using the complete mitogenome or 380 000 years ago using PCG and rRNAs) However, the differences between these two estimations may not be significant considering the various factors that could bias divergence time estimations (García-Moreno, 2004; Lovette, 2004; Galtier *et al.*, 2009), and that we did not estimate error intervals for the divergence time using complete mitogenomes. Regardless, both estimations showed recent divergence times suggesting that the four lineages evolved in the past 500 000 years.

Ours is the first study we are aware of in hummingbirds to use complete mitochondrial genomes for a population-level analysis, and few hummingbird mitogenomes have been published (Morgan-Richards *et al.*, 2008; Prosdocimi *et al.*, 2016; Souto *et al.*, 2016). We searched GenBank for mitogenomes of closely related hummingbirds with more than a single individual sequenced per species to compare their degree of genetic differentiation with the one we observed in *Coeligena*. We only found six mitogenomes for three subspecies of *Amazilia versicolor* (*Amazilia versicolor versicolor* KF624601, NC_024156; *Amazilia versicolor milleri* KP722042, NC033405; and *Amazilia versicolor rondoniae* KP722041, NC_033404; Prosdocimi *et al.*, 2016). Overall, these sequences are more differentiated (5.1% of sites were variable) than our entire data set (1.5%). Although this comparison is far from comprehensive, it does support the idea that the mitogenomes of *Coeligena* lineages are highly similar and their divergence is quite recent relative to other hummingbirds with comparable data, as also indicated by analyses of individual mtDNA genes (Parra *et al.*, 2009; Palacios *et al.*, 2019).

The low, geographically structured and recent genetic divergence of *C. bonapartei* and *C. helianthea* lineages and their discordant patterns of clustering between mitochondrial and nuclear markers suggest a complex evolutionary history where various evolutionary processes may have acted simultaneously. We found that complete mitogenomes of *C. b. bonapartei* and *C. h. helianthea* are undifferentiated even though both subspecies differ strikingly in phenotype and are also distinguishable using nuclear markers. Incomplete lineage sorting could explain this result because the southern mitogenome group exhibited high nucleotide diversity in comparison with *C. b. consita* and *C. h.*

tamai, an unexpected pattern after recent introgression (Krosby & Rohwer, 2009). However, nuclear sorting without mitochondrial sorting would be unlikely because the effective population size of the latter is a fourth of that of the former. Instead, a scenario in which one mitogenome quickly swept through, replacing the mitogenome of the other lineage, seems more likely. Mitochondrial introgression between these lineages was possibly facilitated because they are sympatric at the south of their distribution in the Sabana de Bogotá, and they probably have other points of contact between the slopes of the cordillera to the north, which may have varied through time due to change in habitat conditions (Graham *et al.*, 2010; Flantua *et al.*, 2019).

The divergent mitogenomes of *C. b. bonapartei* individuals ID10 and ID14 were unexpected considering the similarity among all other sequences. These individuals have a mitogenome haplotype which is highly divergent (34 unique variants) and share variants with both the northern (nine variants) and the southern (18 variants) groups. We can reject hybridization with other unstudied taxa as an explanation for these atypical mitogenomes because *ND2* sequences placed these specimens within the clade formed by *C. bonapartei* and *C. helianthea* to the exclusion of *C. b. eos* (Palacios *et al.*, 2019). These atypical sequences may instead be evidence of a relict mitochondrial lineage (i.e. a 'ghost lineage') in *C. b. bonapartei* (Grandcolas *et al.*, 2014; Zhang *et al.*, 2019), perhaps the one that was swept out, which may have remained in isolation on the western slope of the Cordillera Oriental in Boyacá (the Iguaque Massif and surroundings). In this region other atypical patterns in mtDNA have been reported (Guarnizo *et al.*, 2009; Chaves & Smith, 2011; Chaves *et al.*, 2011; Avendaño & Donegan, 2015; Chesser *et al.*, 2020). Another less likely explanation for these atypical sequences may be heteroplasmy and mitochondrial recombination (Rokas *et al.*, 2003; Piganeau *et al.*, 2004; Sammler *et al.*, 2011).

The similarity in the mitogenomes of *C. b. consita* and *C. h. tamai* also appears to be consistent with introgression (independent from the introgression in the nominate subspecies), in this case after genetic and phenotypic differentiation in isolation. Mitochondrial introgression between *C. b. consita* and *C. h. tamai* was possibly facilitated by their geographical proximity and may have happened during a period of greater connectivity of forests in the Pleistocene (Graham *et al.*, 2010; Flantua *et al.*, 2019). Later, both lineages became isolated again and their mitogenomes diverged. The northern mitogenome may thus have evolved within *C. b. consita* and introgressed into *C. h. tamai* in a north to south direction, and such introgression may have further proceeded into *C. b. bonapartei* explaining why individuals ID 12 and 15 have haplotypes more closely related to the northern group.

Mitochondrial introgression may often reflect selection (e.g. adaptive introgression via metabolic efficiency, Ballard & Melvin, 2010; Toews *et al.*, 2014), but may also be due to demographic effects or to asymmetries between sexes in dispersal, mating behaviour and offspring production (Toews & Brelsford, 2012; Rheindt *et al.*, 2014; James *et al.*, 2016; Morales *et al.*, 2017; Harris *et al.*, 2018). We did not find functional changes in PCGs suggesting the mitogenome introgression could be adaptive, although adaptive changes related to substitutions in the control region, or in the 16S rRNA gene in the case of *C. h. tamai*, are still possible. We are unaware of differential dispersal between sexes in *Coeligena*, in which dispersal and breeding biology are poorly known.

In sum, based on our results and earlier work (Palacios *et al.*, 2019) we propose a plausible evolutionary scenario accounting for the discordant patterns of mtDNA, nuclear DNA, and phenotypic variation in *C. bonapartei* and *C. helianthea*. Based on comparisons with the outgroup and other related species (*C. b. eos*, *Coeligena lutetiae*, *Coeligena orina*), the most probable body plumage coloration of the ancestor of our study clade was green with golden/orange underparts. The first lineage to diverge was likely *C. b. consita*, which evolved in the Serranía de Perijá in isolation from the ancestor of the other three lineages, retaining features of the ancestral plumage coloration but diverging in mtDNA. A second divergence event involved sister clades formed by *C. b. bonapartei* and *C. helianthea* (i.e. the common ancestor of both *C. helianthea* subspecies), with the former retaining the ancestral plumage and the latter evolving darker body coloration, a rose belly and an aquamarine rump. These two lineages diverged in phenotype but have an undifferentiated mitogenome owing to incomplete lineage sorting or introgression (except for populations of *C. b. bonapartei* which became isolated on the western slope of the Cordillera Oriental). Subsequently, *C. h. tamai* and *C. h. helianthea* became isolated and diverged slightly in phenotype and genetic markers. Finally, during a period of forest connectivity the mitogenome of *C. b. consita* introgressed into *C. h. tamai* and replaced the existing mitogenome, followed by isolation of these lineages and some divergence in their mitogenomes. Although this is a convoluted historical scenario, it is amenable to testing using genomic data and demographic models (e.g. Aguillon *et al.*, 2018; Kearns *et al.*, 2018; Benham & Cheviron, 2019) and other explanations for patterns of variation would appear even more complex.

In conclusion, low but geographically structured genetic differentiation among lineages of *C. bonapartei* and *C. helianthea* is a general pattern across their mitochondrial genomes despite

their marked phenotypic differences and nuclear phylogenetic relationships. Mitogenomic variation in these lineages seems to reflect geography and demographic history more than the processes shaping their phenotypes and likely most of their nuclear genomes. Studying closely related lineages that diverged recently in complex topographic scenarios, such as the system of *C. bonapartei* and *C. helianthea*, might help to untangle the different effects that various evolutionary mechanisms may have in shaping the divergence between and within genomes. Incomplete lineage sorting, mitochondrial introgression and demographic processes like population bottlenecks, phases of expansion and contraction, and the persistence of relict lineages have likely acted in this system resulting in marked discordance between mtDNA, phenotypes and nuclear markers.

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DATA AVAILABILITY

The data underlying this article are available in the GenBank Nucleotide Database at https://www.ncbi.nlm.nih.gov/genbank/ and can be accessed with accession numbers MT341527 to MT341568.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article on the publisher's website.

Figure S1. Maximum-likelihood phylogenies and haplotype networks for each mtDNA alignment.
Table S1. Specimen data and mitogenome assembly data.